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EUROPEAN PATENT APPLICATION

S.1-3eS0ET8 : Tedmun noitscillqqA (15)

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AT BE CH DE FR GB IT LI LU NE SE Designation of the second of the second

(sa) Process for preparing a macrolide.

(s) A process for preparing tylactone (20-dihydro-20,23-dideoxytylonolide), which has the formula:

CH3-CH3

CH3-CH3

CH3-CH3

by submerged serobic fermentation of Streptomyces tradise NRRL 12188 or a tylactone-producing mutant or recombinant

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PROCESS FOR PREPARING A MACROLIDE

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tylonolide, which will be called tylactone for conpreparation of the macrolide 20-dihydro-20,23-dideoxy-This invention relates to a process for the

venience hereinafter. Tylactone has the structure 1:

acyl derivatives which have structure $\underline{2}$: It is useful in the preparation of related

wherein R and R_{\perp} = an acyl moiety.

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tical to that of tylosin.

macrolide antibiotics.

purified by known techniques.

treatment with acylating agents using methods known in 2-phqroxh dronba to dive acyl ester derivatives by

hours until esterification is substantially complete. about room temperature for from about 1 to about 24

organic solvent (for example, pyridine) at about 0°C to

The derivatives can be prepared by esteri-

acylating agent, such as an acyl anhydride, in an stoichiometric quantity (or a slight excess) of an

as, for example, treatment of the compound with a fication techniques generally known in the art, such

Once formed, the acyl derivatives can be separated and

Acylation can also be achieved by using a mixture of an

acid scavenger) and active esters of organic acids. halides (usually in combination with a base or other

useful as intermediates in the preparation of new

those described by Okamoto et al. in U.S. 4,092,473. carried out enzymatically using procedures such as dicyclohexylcarbodiimide. Acylations can also be organic acid and a dehydrating agent such as N,N'-

Tylactone can be esterified at the 3- and

Typical acylating agents include anhydrides,

The acyl ester derivatives of tylactone are

herein, the stereochemistry of the compounds is idenical assignments are indicated in the structures given antibiotics can be prepared. Although no stereochem-

useful intermediates from which 16-membered macrolide

The compounds of structures $\frac{1}{2}$ and $\frac{2}{3}$ are

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The culture medium used to grow the Streptodesired compound is produced. 52 able culture medium until a substantial amount of the compound under submerged aerobic conditions in a suitstrain of Streptomyces fradiae which produces this Tylactone can be prepared by culturing a maleic, fumaric, malonic and phthalic acids. 50 esters derived from dicarboxylic acids such as succinic, aromatic moiety. Suitable esters also include hemihalogen, nitro, lower alkoxy and the like on the aralkyl- acids optionally bearing substituents such as aryl-, and aralkyl-sulfonic acids, the aryl- and SI acetic, mandelic and 2-thienylacetic acids, and alkyl-, adamantanecarboxylic, benzoic, phenylacetic, phenoxycyclohexanecarboxylic, β-cyclohexylpropionic, lalkoxycarbonic, stearic, cyclopropanecarboxylic, acetic, propionic, butyric, isovaleric, glucuronic, OΤ derived from acids such as formic, acetic, chloro- . Representative suitable esters include those acids, such as sulfuric and phosphoric acids seaders research scids of from 1 to 18 carbon atoms, and of inorganic pererocyclic carboxylic, sulfonic and alkoxycarbonic and including aliphatic, cycloaliphatic, aryl, aralkyl, Useful esters are those of organic acids chromatography and crystallization. mixture by standard procedures such as extraction, The ester derivative can be isolated from the reaction

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preferred. Thus, for example, preferred carbon sources

product isolation, however, certain culture media are

myces fradiae can be any one of a number of media. For

economy in production, optimal yield, and ease of

fragments of the organism to obtain a fresh, actively volume of culture medium with the spore form or mycelial tative inoculum is prepared by inoculating a small is preferable to use a vegetative inoculum. The vege-52 of large tanks with the spore form of the organism, it lag in production commonly associated with inoculation obtained by shake-flask culture. Because of the time preferred. Small quantities of tylactone may be tylactone submerged aerobic fermentation in tanks is 02 For production of substantial quantities of problem. large-scale fermentation media if foaming becomes a such as polypropylene glycol (M.W. about 2000) to add small amounts (i.e. 0.2 ml/L) of an antifoam agent SI requirements of the organism. It may be necessary to the medium in amounts sufficient to meet the growth commonly occur as impurities in other constituents of included in the culture medium. Such trace elements growth and development of the organism should also be OT Essential trace elements necessary for the chloride, carbonate, sulfate, nitrate, and like ions. potassium, sodium, magnesium, calcium, ammonium, customary soluble salts capable of yielding iron, can be incorporated in the culture media are the ς and the like. Among the nutrient inorganic salts which include corn meal, soybean meal, fish meal, amino acids such as soybean oil. Preferred nitrogen sources as dextrin, glucose, starch, and corn meal and oils in large-scale fermentation include carbohydrates such

inoculum is then transferred to a larger tank. The

growing culture of the organism. The vegetative

can also be used. as that used for larger fermentations, but other media medium used for the vegetative inoculum can be the same

The method of this invention comprises

as a major component. only minimal amounts of tylosin, but produces tylactone which produces tylosin. The new microorganism produces chemical mutagenesis of a Streptomyces fradiae strain culturing a new microorganism which was obtained by

This invention also relates to the new micro-

available to the public under the accession number NRRL Street, Peoria, Illinois, 61604, from which it is Research, North Central Region, 1815 North University of the Northern Regional Research Center, Agricultural deposited and made part of the stock culture collection A culture of this microorganism has been organism is also classified as a strain of Streptomyces organism which produces tylactone. The new micro-

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the characteristic of tylactone production are a part binants of Streptomyces fradiae NRRL 12188 which retain All natural and induced variants, mutants and recomgamma rays, and N-methyl-N'-nitro-N-nitrosoguanidine. chemical mutagens, such as ultraviolet light, X-rays, obtained by treatment with various known physical and tants or variants of the NRRL 12188 strain may be subject to variation. For example, recombinants, mucharacteristics of Streptomyces fradiae NRRL 12188 are As is the case with other organisms, the

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of this invention.

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Production of tylactone can be followed

about 30% or above (at 28°C and one atmosphere of

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cent of air saturation for tank production should be For efficient antibiotic production the per-

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processes, sterile air is bubbled through the culture As is customary in aerobic submerged culture

atures of about 28°C.

production of tylactone appears to occur at temperperatures between about 10° and about 40°C. Optimum

S. fradiae WRRL 12188 can be grown at tem-

solvent such as amyl acetate or petroleum ether, con-

both the filtered broth and the mycelial cake.

soluble in the medium in which it is produced.

aerobic fermentation conditions, tylactone can be

Kennedy in J. Chromatographic Science, 16, 492-495 with a UV detection system [see, for example, J.H. broth, using high-performance liquid chromatography during the fermentation by testing samples of the

tration of the fermentation broth and extraction of 1) extraction of the fermentation broth or 2) fil-

conery of tylactone, therefore, can be accomplished by

bility of tylactone in water, it may not be altogether

in the fermentation art. Because of the limited solurecovered from the fermentation medium by methods used

Following its production under submerged

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metabolic statter of mojeties, the metabolic portion or the added sugar mojeties, the metabolic	30
By labeling erener che clares of the	
of labeled compounds for	
whe compound of structure I is	
.autroeodaguratus	
which may be used to obtain the selected strains is	52
same anatata w .nisothia brioducia io oldana	
of Streptomyces strains witch	. •
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service to account they produce of president	
till actone to small shake-flask curtures of end and	20
Common Id DatitauaDI ale Suleats oboum	•
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except that it is blocked in tylactone formation can be	
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• TOTTEM TOTAL FOR THE TOTAL T	
is capable of producing tylosin except care	01
The same of the struct broads of the same	
Two do to B ad upo ws tuebloos	
FORTON SUITS TANDOPOTO P JU PANTITO	
- Figure 16 LYLOSTE 10 by better of	
To very and the DSIEGO of and	•
The compounds of membered macrolide intermediates from which l6-membered macrolide	a T
The compounds of structures 1 and 2 are	
. riele by adsorption chromacographi.	đ
The state of the city of the state of the st	_
entrating the organic phase under vacuum to give	၁
Sylp of munch refer of the	,

pathway of tylosin can be ascertained.

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composition:

provided:

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tion of this invention, the following examples are In order to illustrate more fully the opera-

Shake-flask Fermentation of Tylactone ς Example 1

late a vegetative medium (150 ml) having the following A portion of this solution (0.5 ml) was used to inocu-NRRL 12188 was dispersed in 1-2 ml of sterilized water. A lyophilized pellet of Streptomyces fradiae

54.0 Caco₃ ε.0 Soybean grits ST 5.0 Yeast extract 2.0 Corn steep liquor J.O Ingredient (%) JunomA

Alternatively, a vegetative culture of S. Deicnized water 52.76 Soybean oil (crude)

29°C. for about 48 hours on a closed-box shaker at medium was incubated in a 500-ml Erlenmeyer flask at late the vegetative medium. The inoculated vegetative liquid nitrogen was rapidly thawed and used to inocufradiae NRRL 12188 preserved, in 1-ml volumes, in

having the following composition: was used to inoculate 7 ml of a production medium This incubated vegetative medium (0.5 ml) about 300 rpm.

Water

Lecithin (crude)

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ST	0.0	Y	
	s.0	Soybean oil (crude)	
	E.0	caco ₃	52
	2. 0	Yeast extract	
	2.0	Soybean meal	
	J.0	Corn steep liquor	
(8)	3 nuomA	Ingredient	07
: 11072	arsodwoo bu	growth medium having the following	02
\ <u>_</u>		POSSE B IO I 85 SHELLESS	
		SATIONED LENT OF THE .	
	Ameatum,	Vitalanay beardons;	
mi beredera	arger volu	In order to provide a l	
-woodi to on	(°	rapk Fermentation of Tylacton	ςτ
		closed-box shaker at 300 rpm.	
		The incutated to a 50-ml bottle at 29°C.	
g days on a	muibəm nois	The inoculated fermenta	
		Detonized water	70
	9E.19	Soybean oil (crude)	
	0.5	_€ oɔ₅ɔ	
	2.0	(ин ^ф) _{Уньо} ф	
.,		ИаСЪ	
		Corn djaten	Ġ
	. 6.0	Fish meal	
ere ê :	6.0	Corn meal	
. *	5°T	Beet molasses	
	2.0		
	(%) JunomA	Ingredient	

The pH was adjusted to 8.5 with 50% NaOH solution.

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36	conventional agitators at about 300 rpm.
50	The inoculated production medium was allowed to ferment in a 68-liter tank for about 5 days at a temperature of 28°C. The fermentation medium was aerated with sterile air to keep the dissolved oxygen level between about 30% and 50% and was stirred with
	The pH was adjusted to 7.2 with 50% NaOH solution.
	90.90 Water
	Lecithin
ST	Soybean oil (crude)
	Beet molasses
	0.04 _Q) QHP _Q (NH _Q)
	NaCI
	12.0 21.0 200.50
στ	COLU dincen
-	Corn meal 0.92
	Lish meat
	76 0
	Ingredient (%)

duction medium having the following composition: pared was used to inoculate 40 liters of sterile pro-Incubated second-stage medium (4 L) thus pre-

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incubated in a 68-liter tank for about 47 hours at This second-stage vegetative medium was

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-orbya : 807 , rodrso : carbon, 70%; hydro-	30
about 162-163°C. It has the following approximate	
from hexane or ethyl acetate-hexane and which melts at	
Tylactone is a white solid which crystallizes	
in chloroform is presented in the accompanying drawing.	
The infrared absorption spectrum of tylactone	52
T62-163°C.	
benzene-hexane or hot hexane to give about 2 g, m.p.	
orated under vacuum. Tylactone was crystallized from	
Fractions containing tylactone were combined and evap-	
acetate (9:1) to separate and isolate tylactone.	20
remove lipid substances, then with benzene:ethyl	
detection. The column was first eluted with benzene to	
(3:2) solvent system and conc. sulfuric acid spray for	
layer chromatography, using a benzene:ethyl acetate	
benzene. Elution is monitored by silica-gel thin-	ST
grade 62, Davison Chemical Co.) column, packed with	
chromatographed over a 5.25 x 36 in. silica-gel (Grace,	
dissolved in benzene (5 L). The benzene solution was	
concentrated under vacuum to give an oil. The oil was	
reading at 282 nm but no antimicrobial activity) was	οτ
acetate extract (which has a high optical density	
was extracted with amyl acetate (400 L). The amyl	
by the addition of 2% sodium hydroxide. The filtrate	
Corp.). The pH of the filtrate was adjusted to about 9	
(3% Hyflo Supercel, a diatomaceous earth, Johns Manville	S
described in Example 1, was filtered using a filter aid	
Fermentation broth (1600 L), obtained ha	
Isolation of Tylactone	
Example 2	

spray, either concentrated or dilute (50%), may be used by silica-gel thin-layer chromatography. Sulfuric acid Tylactone can be distinguished from tylosin .abixolluz diethyl ether, petroleum ether, benzene and dimethyl methanol, ethanol, dimethylformamide, chloroform, 52 but is soluble in organic solvents such as acetone, Tylactone is nearly insoluble in water, aqueous dimethylformamide indicates it has no titrata-20 Electrometric titration of tylactone in 66% $(\alpha)_{2}^{25}$ -55.23° (\underline{c} 1, cH_3^{OH}). Tylactone has the following specific rotation: $E_{\text{LC}} = 1000$ mm $E_{\text{LC}} = 100$. tylactone in neutral ethanol exhibits an absorption ST The ultraviolet absorption (UV) spectrum of .(Medium), 820 (very small) and 661 (small). , (medium), 911 (shoulder), 859 (small), 868 (medium), small), 1025 (medium), 984 (very strong), 958 (strong), (strong), 1103 (medium), 1078 (medium), 1049 (very JO (strong), 1284 (medium), 1181 (very strong), 1143 1441 (sponider), 1404 (strong), 1379 (small), 1316 strong), 1626 (small), 1592 (very strong), 1458 (strong), (weak), 2353 (weak), 1709 (very strong), 1678 (very frequencies (cm⁻¹): 3534 (medium), 2924 (strong), 2398 Observable absorption maxima occur at the following in chloroform is shown in the accompanying drawing. The infrared absorption spectrum of tylactone of C₂₃H₃₈O₅ and a molecular weight of about 394. gen, 9.78; oxygen, 20.3%. It has an empirical formula

Table 1.

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approximate Rf values of tylactone are summarized in the chromatography, UV detection is convenient. del plates with a fluorescent background are used in appears initially as a yellow-to-brown spot. If silicafor detection. With this detection system tylactone

Thin-Layer Chromatography of Tylactone

Table 1

[:})	acetate acetate	$e: e \in hY$	Silica gel A = benzen B = benzen	² Medium: b _{Solvent} :	ςτ
	B 0.62	d <u>A</u> 0≥.0 0.0		Compound Tylactone Tylosin	οτ
	ne	RY 18		•	

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Example 3

3,5-Di-O-Acetyltylactone

The $extbf{R}_{ extbf{T}}$ of tylactone in this system is about tography in a benzene:ethyl acetate (4:1) solvent R_f value of about 0.59 on silica-gel thin-layer chromato give 3,5-di-0-acetyltylactone. This compound has an at 60° for 1/2 hour and then concentrated under vacuum (5 ml) was added to the residue; the solution heated 52 then concentrated to dryness under vacuum. Methanol allowed to stand at room temperature for 16 hours and .hydride (4 ml) was added. The resulting mixture was Example 2, was dissolved in pyridine (4 ml). Acetic 20 Tylactone (200 mg), prepared as described in

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Examples 4-7

3,5-Di-O-propionyltylactone, prepared according to the procedure of Example 3, but using propionic anhydride.

3,5-Di-O-isovaleryltylactone, prepared isovaleric anhydride.

3,5-Di-O-benzoyltylactone, prepared according to the procedure of Example 3, but using to the procedure of Example 3, but using pensone.

u-pntAride.
3,5-Di-O-(n-butyryl)tylactone, prepared
according to the procedure of Example 3, but using
according to the procedure of Example 3, but using
according to the procedure of Example 3, but using
according to the procedure.

Example 8

Preparation of Tylosin from Tylactone

A Streptomyces fradise strain which formerly

produced tylosin but which was blocked in macrolide

ring closure was fermented according to the procedure

described in Example 1, Section A, except that a temperature of 28°C was used. Tylactone was added to the

fermentation 48 hours after inoculation. The fermentation was then continued until a substantial amount of

tylosin was produced, i.e. about three additional days.

tylosin was produced, i.e. about three additional days.

The presence of tylosin is determined by testing

samples of the broth against organisms known to be

semples of the broth against organism is

sensitive to tylosin. One useful assay organism is

sensitive to tylosin. One useful assay organism is Staphylococcus aureus ATCC 9144. Bioassay is conveniently performed by an automated turbidometric method, by thin-layer chromatography or by high-performance liquid chromatography with UV detection.

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1. A process for preparing tylactone, or an ester derivative thereof, which comprises cultivating Streptomyces fradise NRRL 12188, or a tylactonemedium containing assimilable sources of carbon, nitrogen, and inorganic salts under submerged aerobic fermentation by esterification.

2. A process according to claim 1 which comprises cultivating Streptomyces fradise NRRL 12188.

3. Streptomyces fradise NRRL 12188.

4. A culture medium which comprises Streptomyces fradise NRRL 12188.

carbon, nitrogen and inorganic salts.

whenever prepared by a process according to either of claims

Tylactone or an ester derivative thereof

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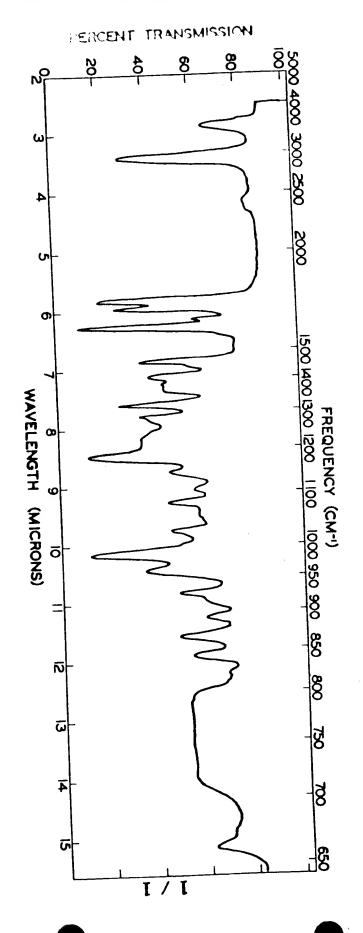
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dates of search Date of completion of the search MOUTH EX The present search report has been drawn up for all claims corresponding document smelled emass of the same patent L: citation for other reasons D: document cited in the E: conflicting application notineval edt T: Theory or principle underlying P: Intermediate document: 19 O: non-written disclosure A: technological background X: particularly relevant CATEGORY OF CITED DOCUMENTS CISP 80/11 C 01 D 313/00 ¥ 61 K 398/18 SEVECHOICAL FIELDS 123 * Complete article * strain, streptomyces fradiae KAfrom a mutant of tylosin-producing membered lactone, protylonolide, characterization of a new 16bns noiselos!" :ARUMO IH20TA2 vol. 28, no. 6, June 1980, pages 1963-1965, edit. by Pharmaceutical Society of Japan 12 R 15 b ၁) 1S B CHEMICAL & PHARMACEUTICAL BULLETIN 1,2,5 0 Χ /80/LL C 01 D 313/00 Y 61 K 398/18 Citation of document with indication, where appropriate, of relevant Relevant misto of DOCUMENTS CONSIDERED TO BE RELEVANT CLASSIFICATION OF THE APPLICATION AM CLA

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The Hague

EUROPEAN PATENT SPECIFICATION

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(3) |uf. Cl.3; A 61 K 31/365,

C15P 17/08 //(C12P17/08, CO1D 313/00'

C15B1/24)

A8.E0.A1 :noitsoffication of patent specification: 14.03.84

(1) Application number: 81302964.2

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- (£) Process for preparing a macrolide.
- (2U) 28234 ensibal zilogensibal 307, East McCarty Street (13) Proprietor: ELI LILLY AND COMPANY
- Colney Lane Morwich NR4 7UH (GB) Cottage No. 1 John Innes Institute Inventor: Seno, Eugene Thomas (SU) 08284 snaibnl ziloqensibnl 7446, Sunset Lane (1) Inventor: Baltz, Richard Henry
- Windlesham Surrey GU20, 6PH (GB) Etl Mood Manor (f) Representative: Hudson, Christopher Mark et al.

- TT6281 8U 08.T0.SO : Yrinoin 9 (8)
- r\S8 nitellu8 S8. r0. a0 (3) Date of publication of application:
- 14,03.84 Bulletin 84,111 (a) Publication of the grant of the patent:
- BE CH DE FR GB IT LI LU NL SE (b) Designated Contracting States:
- :befic secrences cited:

producing strain, streptomyces fradiae KA-427 protylonolide, from a mutant of tylosincharacterization of a new 16-membered lactone. bns noitslos!" : ARUMO IH2OTA2 edit. by Pharmaceutical Society of Japan, ,8361-5361 pages 1980, pages 1963-1965, CHEMICAL & PHARMACEUTICAL BULLETIN,

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paid. (Art. 99(1) European patent convention). be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall Note: Within nine months from the publication of the mention of the grant of the European patent, any person may

excess) of an acylating agent, such as an acyl pound with a stoichiometric quantity (or a slight such as, for example, treatment of the comfication techniques generally known in the art. The derivatives can be prepared by estign tone for convenience hereinafter. Tylactone has separated and purified by known techniques dideoxytylonolide, which will be called tylac-Once formed, the acyl derivatives can by preparation of the macrolide 20-dihydro-20,23described by Okamoto et al. in U.S. 4,092,473 This invention relates to a process for the

phosphoric acids.

the structure 7:

derivatives which have structure 2: It is useful in the preparation of related acyl

wherein R and $R_1 = an$ acyl moiety. г

try of the compounds is identical to that of the structures given herein, the stereochemisno stereochemical assignments are indicated in macrolide antibiotics can be prepared. Although useful intermediates from which 16-membered The compounds of structures 1 and 2 are

Tylactone can be esterified at the 3- and 5-'uisoiki

paration of new macrolide antibiotics. tylactone are useful as intermediates in the preknown in the art. The acyl ester derivatives of treatment with acylating agents using methods hydroxyl groups to give acyl ester derivatives by

enzymatically using procedures such as those carbodiimide. Acylations can also be carried out hydrating agent such as N,N'-dicyclohexylusing a mixture of an organic acid and a deorganic acids. Acylation can also be achieved by other acid scavenger) and active esters of halides (usually in combination with a base or Typical acylating agents include anhydrides,

inorganic salts which can be incorporated in the meal and amino acids. Among the nutrient sources include corn meal, soybean meal, fish broduced. si brinomoo besired compound is ditions in a suitable culture medium until a subthis compound under submerged serobic con-

strain of Streptomyces fradiae which produces Tylactone can be prepared by culturing a maleic, fumaric, malonic and phthalic acids. derived from dicarboxylic acids such as succinic, moiety. Suitable esters also include hemi-esters halogen, nitro and lower alkoxy on the aromatic acids optionally bearing substituents such as stalkyl-sulfonic acids, the aryl- and stalkyland 2-thienylacetic acids, and alkyl-, aryl-, and zoic, phenylacetic, phenoxyacetic, mandelic hexylpropionic, 1 - adamantanecarboxylic, bencarboxylic, cyclohexanecarboxylic, β - cyclocuronic, alkoxycarbonic, stearic, cyclopropanechloroacetic, propionic, butyric, isovaleric, giuderived from acids such as formic, acetic

Representative suitable esters include those

and of inorganic acids, such as sulfuric and carbonic scids of from 1 to 18 carbon atoms. heterocyclic carboxylic, sulfonic and alkoxyincluding aliphatic, cycloaliphatic, aryl, aralkyl. Useful esters are those of organic acids

standard procedures such as extraction be isolated from the reaction mixture by substantially complete. The ester derivative can ture for from 1 to 24 hours until esterification is

pyridine) at about 0°C to about room temperaanhydride, in an organic solvent (for example,

chromatography and crystallization.

carbonate, sulfate and nitrate ions. magnesium, calcium, ammonium, chloride; capable of yielding iron, potassium, sodium, culture media are the customary soluble saits and oils such as soybean oil. Preferred nitrogen such as dextrin, glucose, starch, and com meal large-scale fermentation include carbohydrates Thus, for example, preferred carbon sources in however, certain culture media are preferred. optimal yield, and ease of product isolation, number of media. For economy in production, Sueptomyces tradiae can be any one of a The culture medium used to grow the

eniuper function of the growth require mpurites in other constituents of the medium in Such trace elements commonly occur as should also be included in the culture medium: growth and development of the organism Essential trace elements necessary for the

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graphic Science, 16, 492-495 (1978)]. [see, for example, J. H. Kennedy in J. Chromatochromatography with a UV detection system

-qiosbs yd berinned ye may be punfied by adsorpphase under vacuum to give crystals or an oil. If petroleum ether, concentrating the organic with a suitable solvent such as amyl acetable or the broth (generally without pH adjustment) cation of the filtered broth involves extracting Processes. A preferred technique for purifirechniques may be used in the sampinnast filtered broth and the mycelial cake. A variety of fermentation broth and extraction of both the of the fermentation broth or S in the figure of the therefore, can be accomplished by 1) extraction which it is produced. Recovery of tylactone; ni mulbem ant ni aldulor rangether ad ton yem of the limited solubility of tylactone in water, it. methods used in the fermentation art. Because be recovered from the fermentation medium by aerobic fermentation conditions, tylactone can Following its production under submerged

The compounds of structures I and 2 are tion chromatography.

tylosin by adding it to a growing culture of a bioexample, tylactone (1) can be bioconverted to macrolide antibiotics can be prepared. For usetul intermediates from which 16-membered

capable of producing tylosin except that it is strain which either produces tylosin itself or is microorganism can be a Streptomyces fradiae converting microorganism The bioconverting

to small shake-flask cultures of the selected sur-These strains are identified by adding tylactone strains are also unable to produce tylactone. tylosin are further screened to determine which Those survivors which are unable to produce for those which are unable to produce tylosin. strain a mutagen and screening survivors can be obtained by treating a tylosin-producing except that it is blocked in tylactone formation A strain which is capable of producing tylosin blocked in tylactone formation.

nitrosoguanidine. -ontin-'N-lydtem-N si anients betoelee edt A typical mutagen which may be used to obtain strains which are capable of producing tylosin. NRRL 2703 are examples of Streptomyces Streptomyces fradiae strains NRRL 2702 and vivors to determine if they produce tylosin.

the metabolic pathway of tylosin can be tylactone portion or the added sugar moieties, for metabolic studies. By labeling either the useful in the preparation of labeled compounds The compound of structure 1 is especially

In order to illustrate more fully the operation ascertained.

:bebivorq of this invention, the following examples are

3.0) notiulos sint to notition A state besilises NRRL 12188 was dispersed in 1-2 ml of A lyophilized pellet of Streptomyces fradiae A. Shake-flask Fermentation of Tylactone Example 1

the broth, using high-performance liquid to saldmes gnitsat yd noiternamiet ant gnirub Production of tylactone can be followed

28°C and one atmosphere of pressure). production should be about 30% or above (at production the percent of air saturation for tank the culture medium. For efficient antibiotic

culture processes, sterile air is bubbled through

As is customary in aerobic submerged 28°C. appears to occur at temperatures of about 40°C. Optimum production of tylactone

temperatures between about 10° and about

S fradiae NRRL 12188 can be grown at invention. teristic of tylactone production are a part of this

fradiae NRRL 12188 which retain the charac-

mutants and recombinants of Streptomyces guanidine. All natural and induced variants,

gamma rays, and N-methyl-N'-nitro-N-nitroso-

mutagens, such as ultraviolet light, X-rays,

with various known physical and chemical

12188 strain may be obtained by treatment recombinants, mutants or variants of the NRRL

12188 are subject to variation. For example,

characteristics of Streptomyces fradise NRRL

from which it is available to the public under the

North University Street, Peoria, Illinois, 61604, tural Research, North Central Region, 1815

Northern Regional Research Center, Agricul-

part of the stock culture collection of the

microorganism has been deposited and made

strain of Streptomyces fradiae. A culture of this

amounts of tylosin, but produces tylactone as a

The new microorganism produces only minimal myces fradiae strain which produces tylosin.

obtained by chemical mutagenesis of a Streptoculturing a new microorganism which was

The method of this invention comprises

that used for larger fermentations, but other

for the vegetative inoculum can be the same as

transferred to a larger tank. The medium used

organism. The vegetative inoculum is then

a fresh, actively growing culture of the

or mycelial fragments of the organism to obtain

volume of culture medium with the spore form

lisms a gnisculoni by inoculating a small

to use a vegetative inoculum. The vegetative

the spore form of the organism, it is preferable

associated with inoculation of large tanks with

Because of the time lag in production commonly

may be obtained by shake-flask culture.

tanks is preferred. Small quantities of tylactone

tylactone submerged aerobic fermentation in

2000) to large-scale fermentation media if

agent such as polypropylene glycol (M.W. about

meotitre as to (J\lm 2.0 .e.i) stanoms lisms bbs

ments of the organism. It may be necessary to

For production of substantial quantities of

The new microorganism is also classified as a

accession number NRRL 12188.

major component.

media can also be used.

foaming becomes a problem.

As is the case with other organisms, the

381. 76	₩ater
310.0	Lecithin (crude)
9.0	Soybean oil (crude)
£.0	c ₀ O3e3
· 6.0	Yeast extract
3. 0	Soybean meal
0.1	Corn steep liquor
(%) InvomA	Ingredient

The pH was adjusted to 8.5 with 50% NaOH ion.
This second-stage vegetative medium

This second-stage vegetative medium was incubated in a 68-liter tank for about 47 hours at 29°C.
Incubated second-stage medium (4 L) thus

Incubated second-stage medium (4 L) thus prepared was used to inoculate 40 liters of sterile production medium having the following composition:

06.06	Nater
60.0	Lecithin
3.15	Soybean oil (crude)
2.10	sessiom teed
4 0.0	,04H _s (₄ HN)
01.0	NaCI
12.0	CaCO ₃
26.0	Corn gluten
۲ ۵ .۱	Corn meal
26.0	Fish meal
(%) InvomA	Ingredient

The PH was adjusted to 7.2 with 50% NaOH solution.

The inoculated production medium was allowed to ferment in a 68-liter tank for about 5 days at a temperature of 28°C. The fermentation medium was aerated with sterile air to keep the dissolved oxygen level between about 30% and 50% and was stirred with conventional agitators at about 300 rpm.

mul) was used to inoculate a vegetative medium:
(150 ml) having the following composition:
Ingredient (%)

in-the and a Movitedaeth	- "	_
Deionized water	32.76	
Soybean oil (crude)	94.0	
coco,	દ .0	
Soybean grits	8.0	
Yeast extract	3 .0	
Corn steep liquor	0.1	
Ingredient	(%) InvomA	

Alternatively, a vegetative culture of 5 fradiae NRRL 12188 preserved, in 1-ml volumes, in liquid nitrogen was rapidly thawed and used to inoculate the vegetative medium. The inoculated vegetative medium was incubated in a 500-ml Erlenmeyer flask at 29°C, for about 48 hours on a closed-box shaker at about 300 rpm.

This incubated vegetative medium (0.5 ml) was used to inoculate 7 ml of a production medium having the following composition:

· -	
Deionized water	96.16
Soybean oil (crude)	0.8
CaCO ₃	2.0
POdHz(hW)	₽0.0
NaCI	r.o
Corn gluten	6·O
Fish meal	6.0
Com meal	3.1
sesselom teed	2.0
Ingredient	(%) InnomA

The inoculated fermentation medium was incubated in a 50-ml bottle at 29°C. for about 6 days on a closed-box shaker at 300 rpm.

B. Tank Fermentation of Tylactone of In order to provide a larger volume of inoculum, 60 ml of incubated vegetative medium, prepared in a manner similar to that described in section A, was used to inoculate 38 L of a second-stage vegetative growth medium having the following composition:

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Example 2

Isolation of Tylactone

containing tylactone were combined and evaporated under vacuum. Tylactone was crysto separate and isolate tylactone. Fractions stances, then with benzene:ethyl acetate (9:1) eluted with benzene to remove lipid subacid spray for detection. The column was first acetate (3:2) solvent system and conc. sulfuric layer chromatography, using a benzene:ethyl benzene. Elution is monitored by silica-gel thin-Davison Chemical Co.) column, packed with 5.25 x 36 in. silica-gel (Grace, grade 62, benzene solution was chromatographed over a The oil was dissolved in benzene (5 L). The was concentrated under vacuum to give an oil. reading at 282 nm but no antimicrobial activity) scetate extract (which has a high optical density extracted with amyl acetate (400 L). The amyl of 2% sodium hydroxide. The filtrate was filtrate was adjusted to about 9 by the addition earth, Johns Manville Corp.). The pH of the filter aid (3% Hyflo Supercel, a diatomaceous described in Example 1, was filtered using a Fermentation broth (1600 L), obtained as

tone in chloroform is presented in the accom-The infrared absorption spectrum of tylacgive about 2 g, m.p. 162-163°C. to from benzene-hexane or hot hexane to

panying drawing.

oxygen, 20.3%. It has an empirical formula of composition: carbon, 70%; hydrogen, 9.7%; following approximate percentage elemental melts at about 162-163°C. It has the from hexane or ethyl acetate-hexane and which Tylactone is a white solid which crystallizes

lactone in chloroform is shown in the orocon on original observable absorption The infrared absorption spectrum of ty-C₂₃H₃₈O₅ and a molecular weight of about 394.

(strong), 923 (medium), 911 (shoulder), 859 (strong), 868 (medium), 840 (medium), 820 1025 (medium), 984 (very strong), 958 (medium), 1078 (medium), 1049 (very small), 1379 (small), 1316 (strong), 1284 (medium), 1079 (strong), 1103 (strong), 1103 1458 (strong), 1441 (shoulder), 1404 (strong), (very strong), 1626 (small), 1592 (very strong), (Megk), 2353 (Wedk), 1709 (very strong), 1678 maxima occur at the following frequencies (cm⁻¹): 3534 (medium), 2924 (strong), 2398

The ultraviolet absorption (VU) spectrum of (llems) f33 bns (llems viev)

(E1% = 560). mn S8S tuode te mumixem noitqroade tylactone in neutral ethanol exhibits an

Tylactone has the following specific rotation:

 $[\alpha]_{2e}^{\Sigma e} - 22.23^{\circ} (c \ 1) \text{ CH}_{3}\text{OH}).$

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using n-butyric anhydride.

using benzoic anhydride.

using isovaleric anhydride.

using propionic anhydride.

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3,5-Di-O-Acetyltylactone

Medium: Silica gel

according to the procedure of Example 3, but 3,5 - Di - O - (n - butyryl)tylactone, prepared

according to the procedure of Example 3, but

according to the procedure of Example 3, but

according to the procedure of Example 3, but

solvent system. The Rt of tylactone in this tography in a benzene:ethyl acetate (4:1)

of about 0.59 on silica-gel thin-layer chroma-

acetyltylactone. This compound has an R, value

centrated under vacuum to give 3,5-di-O-

tion heated at 60° for 1/2 hour and then con-(5 ml) was added to the residue; the solu-

trated to dryness under vacuum. Methanol temperature for 16 hours and then concen-

moor te bnets of bewolle sew arutxim gnitlusar

Acetic anhydride (4 ml) was added. The

Example 2, was dissolved in pyridine (4 ml).

Example 3

bSolvent: A=benzene:ethyl acetate (4:1)

Tylactone (200 mg), prepared as described in

B=benzene:ethyl acetate (3:2)

3,5 - Di - O - benzoyltylactone, prepared

3,5 - Di - O - isovaleryltylactone, prepared

3,5 - Di - O - propionyltylactone, prepared Examples 4---7

aqueous dimethylformamide indicates it has no Electrometric titration of tylactone in 66%

T 3J8AT

enie	SV 1H	
8	٩V	punodmo
29.0	09.0	Tylactone
0.0	0.0	nisolvT

nisolγT	0.0	0.0
Tylactone	09.0	29.0
DunodmoJ	q∀	8

gisolvT	. 00	00
Tylactone	09.0	29.0
punodwoo	۹∀	8
	BV 17	

Thin-Layer Chromatography of Tylactone

Selfer Control .f əldsT ni bəsinsmmus rescent background are used in the chrones tography, UV detection is convenients the approximate Rt values of tylacityms, are summarized in Table 1. system tylactone appears initially as a yellow to-brown spot. If silica-gel plates with a fluctorby silica-gel thin-layer chromatography, Suffuge acid spray, either concentrated or dilute (508) may be used for detection. With this detection may be used for detection.

Tylactone can be distinguished from tylosing and dimethyl sulfoxide. form, diethyl ether, petroleum ether, benzene methanol, ethanol, dimethylformamide, chloro soluble in organic solvents such as acetone,

Tylactone is nearly insoluble in water, but is titratable groups.

Revendications

1. Procédé de préparation de tylactone ou d'un de ses dérivés esters, caractérisé en ca qu'il consiste à cultiver la souche Streptomyces tradise NRRL 12188, un de ses mutants ou recombinants producteurs de tylactone, dans un milieu de culture contenant des sources assimilables de carbone, d'azote et de sels inorbables de carbone, d'azote et de termentation ganiques dans des conditions de fermentation aérobie submergée pour produire de la tylactone, cette culture étant éventuellement suivie d'une estérification.

2. Procédé suivant la revendication 1, caractérisé en ce qu'il consiste à cultiver la souche Streptomyces fradiae NRRL 12188.

Patentansprüche

1. Verfahren zur Herstellung von Tylacton oder einem Esterderivat hiervon, dadurch gekennzeichnet, daß man Streptomyces fradiae NARL 12188 oder eine tylactonbildende Mutante oder Rekombinante hiervon in einem Kulturmedium, das assimilierbare Quellen für Kohlenstoff, Stickstoff und anorganische Salze enthält, unter submersen aeroben Fermentationsbedingungen und Bildung von Tylacton züchtet und gegebenenfalls dann eine Veresterung vornimmt.

estering vormining.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß man Streptomyces fradiae NRRL 12188 züchtet.

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bgraphy or by high-performance liquid chromaurbidometric method, by thin-layer chromaconveniently performed by an automated Staphylococcus aureus ATCC 9144. Bioassay is to tylosin. One useful assay organism is broth against organisms known to be sensitive tylosin is determined by testing samples of the about three additional days. The presence of stantial amount of tylosin was produced, i.e. fermentation was then continued until a subthe fermentation 48 hours after inoculation. The ture of 28°C was used. Tylactone was added to Example 1, Section A, except that a temperaaccording to the procedure described in in macrolide ring closure was fermented merly produced tylosin but which was blocked - Streptomyces fradiae strain which for-Preparation of Tylosin from Tylactone

emislO

tography with UV detection.

1. A process for preparing tylactone, or an ester derivative thereof, which comprises cultivating Streptomyces tradise NRRL 12188, or a tylactone-producing mutant or recombinant thereof, in a culture medium containing assimilable sources of carbon, nitrogen, and incoganic salts under submerged serobic fermentation conditions to produce tylactone, mentation conditions to produce tylactone, followed, optionally, by esterification.

2. A process according to claim 1 which comprises cultivating Streptomyces fradiae NRRL 12188.

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